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Siderophore Production by a Marine *Pseudomonas aeruginosa* and Its Antagonistic Action Against Phytopathogenic Fungi

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Abstract

A marine isolate of fluorescent *Pseudomonas* sp. having the ability to produce the pyoverdine type of siderophores under low iron stress (up to 10 μ*M* iron in the succinate medium) was identified as *Pseudomonas aeruginosa* by using BIOLOG Breathprint and siderotyping. Pyoverdine production was optimum at 0.2% (w/v) succinate, pH 6.0, in an iron-deficient medium. Studies carried out in vitro revealed that purified siderophores and *Pseudomonas* culture have good antifungal activity against the plant deleterious fungi, namely, *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus oryzae*, *Fusarium oxysporum*, and *Sclerotium rolfsii*. Siderophore-based maximum inhibition was observed against *A. niger*. These in vitro antagonistic actions of marine *Pseudomonas* against phytopathogens suggest the potential of the organism to serve as a biocontrol agent.

Index Entries: Marine fluorescent *pseudomonas*; siderophores; phytopathogens; biocontrol agent; pyoverdine.

Introduction

Currently, microbes from terrestrial sources are employed as bioinoculants and biocontrol agents for agricultural use, but the potential for

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synthesis of several novel secondary metabolites by marine microorganisms has been recognized *(1)*. Over a period of two decades, numerous studies on detection of secondary metabolites produced by marine isolates have been conducted. However, large-scale production of metabolites was not attempted. Interest in marine biotechnology has been growing in recent years, which may open new opportunities for using and managing biologic resources from the seas and other bodies of water *(2)*. The use of open ocean bacteria has also been reported for the production of siderophores (iron chelators) with exceptionally high affinities for iron *(3)*, though open ocean bacteria may not need iron as much as terrestrial bacteria because they have substituted non-iron-containing compounds to do the work of those containing iron. The best-documented case of this is the use of flavodoxin instead of ferridoxin by various cyanobacteria *(4)*.

Control of plant diseases has remained a challenge to mankind. Conventional strategies of disease control were replaced with the use of chemical pesticides. However, these pesticides affected soil fertility and the ecosystem *(5)*. To overcome this problem, biocontrol agents have been well exploited *(6–9)*. Fluorescent pseudomonads are known to produce a variety of secondary metabolites that have a dual effect toward plants: plant growth promotion and suppression of phytopathogens *(10–12)*.

The present study attempted to identify and characterize a marine isolate, and to optimize and maximize its siderophore production, its purification and in vitro antagonistic action against fungal plant pathogens, and its interaction with other rhizobacteria.

Materials and Methods

Organism and Culture Conditions

A marine isolate from seawater of Goa was isolated on seawater agar and cetrimide agar. Further, the isolate was identified according to *Bergey's Manual of Systematic Bacteriology (13)* and then confirmed with the help of Belgian Coordinated Collections of Microorganisms, commonly referred to as BIOLOG Breathprint, using BIOLOG GN (USA) microtiter plates designed to test the ability of an organism to oxidize 95 different carbon sources.

The results of the BIOLOG breathprint were reconfirmed by using siderotyping, a novel method of identifying fluorescent pseudomonads based on the type of pyoverdine (Pvd) produced, where casamino acid culture supernatant was analyzed for its Pvd content by isoelectric focusing (IEF) (Bio-Rad, Hercules, CA) according to a method described by Meyer et al. *(14)*.

Culture was maintained on nutrient agar slants and King's B medium throughout the study. All chemicals were of analytical and pure grade and were procured from Hi Media (Mumbai) and Sigma (St. Louis, MO).

Preparation of Inoculum

Inoculum was raised using actively growing culture (18–24 h) in King's B medium in a rotary incubator shaker at 180 rpm and 28°C.

Medium and Growth Conditions for Siderophore Production

An iron-free succinate medium (SM) (pH 7.0) consisting of the following components was used for siderophore production: 6 g/L of K_2HPO_4, 3 g/L of KH_2PO_4, 0.2 g/L of $MgSO_4 \cdot 7H_2O$, 1 g/L of $[NH_4]_2SO_4$, and 4 g/L of succinic acid. Previously developed 1% (v/v) inoculum was inoculated in iron-deficient SM and incubated as just described *(15)*. After every 12 h of incubation, the culture was centrifuged at 10,000*g* for 10 min at 4°C, and the cell-free supernatant was subjected to qualitative and quantitative determination/confirmation of siderophores with the help of Universal Chrome Azurol S (CAS) assay and CAS-shuttle assay *(16,17)*. The supernatant was also scanned on a spectrophotometer (UV-Visible 1601; Shimadzu, Kyoto, Japan) in the range of 200–500 nm *(18)*. Similarly, the supernatant was analyzed by Csaky's *(19)* and Arnow's *(20)* assay to determine the type of siderophore produced by the organism.

Optimization of Siderophore Production

Siderophore production by test organism was checked as a function of different influential parameters: succinate concentration, pH, and iron concentration. Each set with a standard variable was inoculated with 1% (v/v) active culture and incubated at 28°C and 180 rpm for 40 h. To check the influence of substrate concentration, succinate was varied in the range of 0.1–0.5% (w/v) with an interval of 0.1%, and pH was varied in the range of 2.0–10.0. Iron was incorporated into the SM in the concentration range of 1–100 µ*M*.

Extraction and Purification of Siderophores

Siderophores were extracted from culture grown in iron-free succinate medium according to Stintzi and Meyer *(21)*. Siderophore containing supernatant were purified by subjecting the acidified (pH 6.0) culture-filtrate through an XAD-2 column (Aldrich) and eluting with 50% methanol. The brown fraction was concentrated and dried under vacuum on a rotary evaporator and checked for CAS.

In Vitro Suppression of Phytopathogenic Fungi

The antifungal activity of siderophore formulation was tested in vitro by agar plate assay on potato dextrose agar and King's B medium. Different soilborne plant pathogenic fungi—*Fusarium oxysporum* NCIM 1008, *Sclerotium rolfsii* NCIM 1084, *Aspergillus niger* NCIM 1025, *Aspergillus flavus* NCIM 650, and *Aspergillus oryzae* NCIM 122—were tested by two different methods advocated by Manwar *(22)*. In the first method, the fungal spore

suspension was mixed with melted, cooled agar and poured into sterile Petri plates. After hardening of the medium, sterile paper disks soaked in cell-free culture filtrate were placed on the plates. In the second method, 100 µL of filter-sterilized culture filtrate was spread on the solidified agar plates using a spreader, and the fungal culture was spot inoculated at the center. Control plates were kept for both methods without addition of culture supernatant and siderophore. All the plates were incubated at room temperature for 48 h and the results were recorded.

Simultaneously, standard rhizobacteria—*Rhizobium* sp., *Bradyrhizobium japonicum*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Azotobacter vinelandii* (local isolates from soil)—were also tested in a similar manner in order to determine the effect of *Pseudomonas* and siderophores on their growth.

Results and Discussion

The oceans offer abundant resources for research and development, yet the potential of this domain as the basis for new biotechnologies remains largely unexplored. Indeed, the vast majority of marine organisms are yet to be identified. For known organisms, there is insufficient knowledge to permit their intelligent management and application. Marine organisms are of great scientific interest because they often possess unique structures, metabolic pathways, reproductive systems, and sensory and defense mechanisms. Major classes of the earth's organisms are exclusively marine, so the oceans represent sources of unique genetic information *(23)*.

Characterization of Marine Isolate

Keeping the aforementioned aspects in mind, we obtained a marine isolate from the coastal waters of Goa. The isolate tolerated a salt concentration up to 6%. However, it was also capable of exhibiting normal growth at a salt concentration ranging from 0.5 to 1.0% incorporated in a medium preparation such as nutrient broth/agar. It was further identified as a member of the fluorescent pseudomonad group as per *Bergey's Manual of Systematic Bacteriology*. In addition, it was confirmed by Breathprint using BIOLOG GN microtiter plates as *P. aeruginosa* ID 4365. This identification based on the BIOLOG GN method was reconfirmed by using the Pvd-based siderotyping method reported by Meyer et al. *(14)*, and the isolate was characterized as *P. aeruginosa* Sidrovar II, with p*I* values of 8.5 and 7.6 similar, to those of *P. aeruginosa* ATCC 27853 (Fig. 1).

Pvd Production and Characterization

Maximum and sustained siderophore production up to 72 h was noted at a 0.2% (w/v) succinate concentration (Fig. 2A). Higher concentrations (>0.2% [w/v]) of succinate favored biomass accumulation with decreased siderophore production. Figure 2B confirms the optimal pH for siderophore production by *P. aeruginosa* as 6.0. pH values of 2.0 and 4.0 were found to

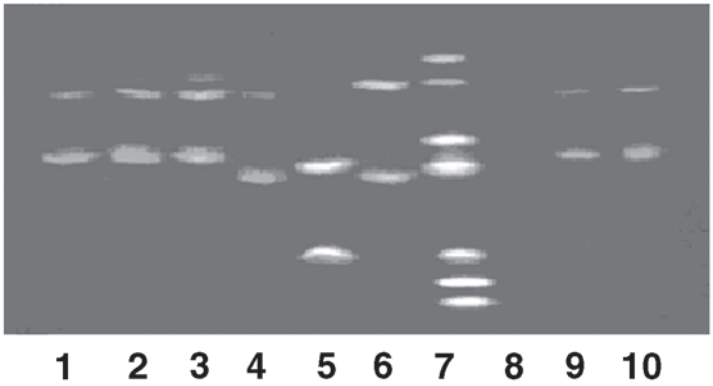

Fig. 1. IEF analysis of Pyoverdine produced by *P. aeruginosa* ID 4365. *Lanes 1, 2, 9,* and *10*: ID 4365; *lane 3*: *P. aeruginosa* ATCC 27853; *lane 4*: *P. fluorescens* 18; *lane 5*: *P. aeruginosa* pa 6; *lane 7*: standard; *lane 8*: empty.

be unsuitable for growth and siderophore production. Higher pH favored growth of the organism but not siderophore production. A pH value of 9.0 was found to be optimal for growth, which may be owing to the marine origin of the test organism.

At 20 and >20 μ*M* iron concentrations, complete retardation of siderophore production was observed, as depicted in Fig. 2C. This finding is in agreement with observations recorded earlier by Presmark et al. *(24)*. Siderophore production was found to be inversely proportional to Fe^{3+} concentration, whereas growth was directly proportional to Fe^{3+} concentration. Iron-limiting conditions imparted organisms to produce siderophores for competitive iron uptake.

Siderophores were isolated and purified in free ligand form according to the method reported by Stintzi and Meyer *(21)*. One liter of culture filtrate yielded 240 mg of pure desferric form of siderophore (vacuum-dried weight).

The absorption maxima of cell-free supernatant at 404 nm (Fig. 3) and instant appearance of golden yellow color after reacting with CAS reagent confirmed production of the Pvd type of siderophore, with 60.81% siderophore units as compared to the reference containing an equal amount of uninoculated SM and CAS assay solution.

Spectrophotometric analysis of the siderophore sample showed a sharp peak at 404 nm, which is characteristic of the Pvd type of siderophore *(15)*. Further, the Csaky's and Arnow's assays were performed, which revealed that the Pvd sample gave a strong positive Csaky test, indicating the presence of the hydroxamate type of siderophore.

In Vitro Phytopathogen Suppression

The zone of inhibition against pathogenic fungi produced by the organism shows the ability of both *Pseudomonas* and its (siderophore) to inhibit fungal plant pathogens (Table 1). The degree of inhibition varied

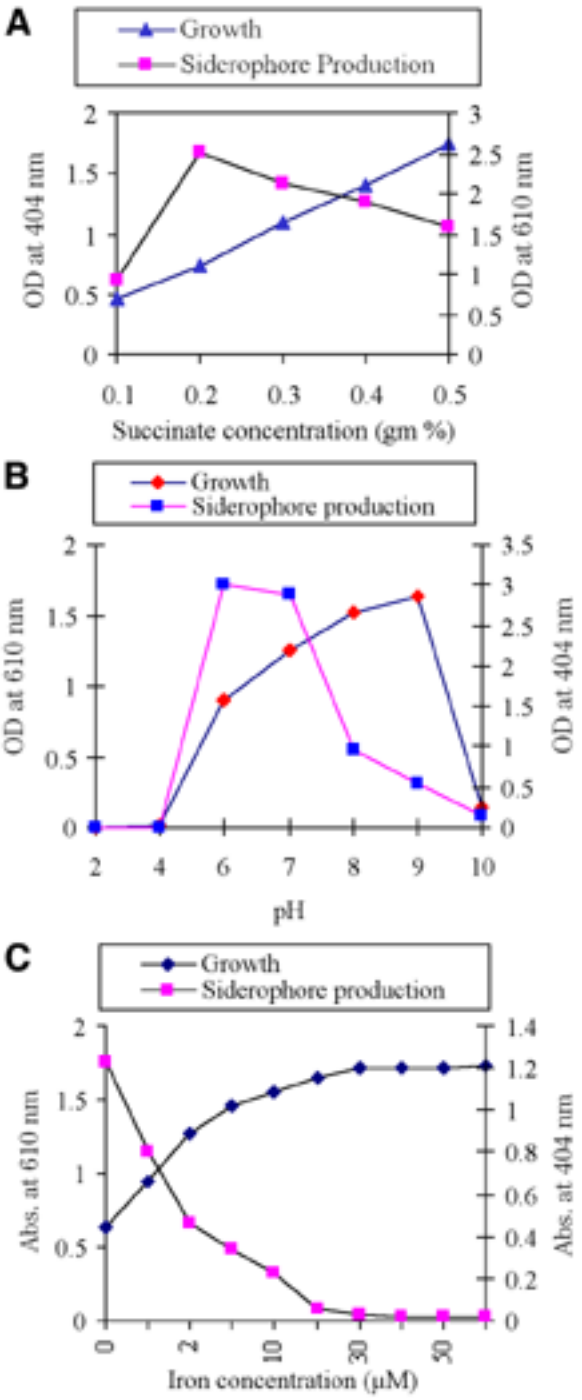

Fig. 2. **(A)** Influence of succinic acid concentration on growth and siderophore production by *P. aeruginosa*; **(B)** effect of pH on growth and siderophore production by *P. aeruginosa* at various pH values in iron-free succinate medium after 40 h of incubation; **(C)** influence of iron concentration on growth and siderophore production.

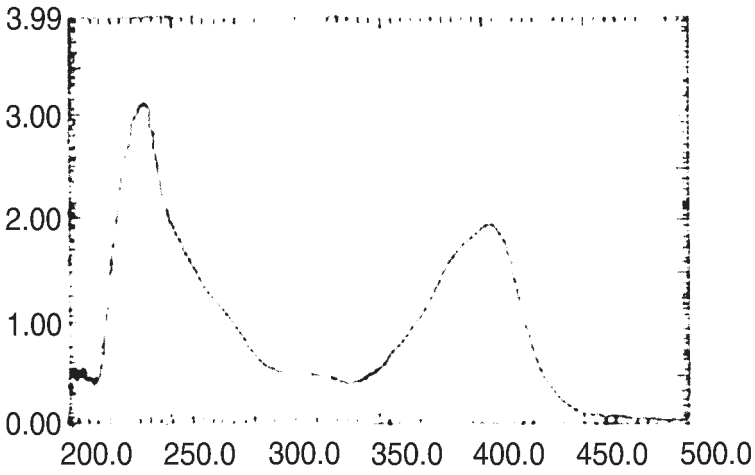

Fig. 3. Spectrophotometric profile of siderophores from marine *P. aeruginosa*.

Table 1
In Vitro Antibiosis Produced by Marine Isolate Against Plant Pathogens[a]

	Zone of inhibition (mm)			
	Potato dextrose agar		King's B medium	
Fungal strain	XAD-purified siderophore	Supernatant	XAD-purified siderophore	Supernatant
A. flavus	7	9	10	13
A. niger	10	10	30	35
A. oryzae	4	6	16	17
F. oxysporum	6	9	11	17
S. rolfsii	4	8	20	24

[a]Antibiosis is as zone of inhibition (mm). Values are averages from duplicate culture.

with the test organism and the medium used. The zone of inhibition was wider in most cases on King's B medium, as reported earlier *(8)*.

Our study showed that the marine *P. aeruginosa* was capable of producing siderophore when grown under iron-starvation conditions like other pseudomonads. It was also noted that synthesis of Pvds in fluorescent *Pseudomonas* was repressed by exogenous addition of Fe^{3+}, as reported earlier *(25,26)*. The antagonistic action exerted by siderophores and certain secondary metabolites seems to have a good cumulative effect. It was revealed that the supernatant of the marine isolate had a greater inhibition activity against the fungal pathogens as compared to the pure siderophores, as depicted in Table 1. These results are in accordance with results obtained by other laboratories *(6,7,27)*. The supernatant retained its inhibitory activity against *A. niger* even after 10-fold dilution. It was also revealed that the degree of inhibition varied with the medium used. King's B medium was

found to show maximum activity against all the tested phytopathogens. The reason for such a difference in the degree of inhibition might be owing to the varying degree of diffusion levels of siderophore/supernatant in the medium. *Pseudomonas* culture or siderophore-rich culture supernatant did not show growth inhibitory action against tested rhizobacteria, as reported earlier by our laboratory *(28)*, which leads us to confirm that neither *Pseudomonas* nor siderophores of *Pseudomonas* have any adverse effect on the rhizobacteria; on the contrary, *Pseudomonas* culture/culture filtrate promoted growth of the *Azotobacter* and *Rhizobium* spp. under study, and, therefore, such a system seems to be ecofriendly.

These results are promising for the development of siderophore-based or fluorescent pseudomonads–based biocontrol agents. O'Sullivan and O'Gara *(10)* have proposed a mechanism of siderophore-mediated suppression of plant pathogens. According to this mechanism, siderophore-producing *Pseudomonas* are capable of utilizing iron-siderophore chelate (Fe-S), through a specific receptor, whereas the plant deleterious microorganisms are deprived of or unable to accept such a chelate. From our observations and results, we conclude that the *P. aeruginosa* of marine origin has dominant characteristics over terrestrial rhizobacteria, because it is nutritionally versatile, has rapid growth rates, produces a higher concentration of siderophores along with other secondary metabolites, has antifungal activity against certain plant deleterious fungi, and is ecofriendly. Thus, this organism has the potential to act as an efficient biocontrol agent.

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